Cellular signalling: **Stressing the importance of PIP_3**

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The lipid second messenger PIP_3 was previously thought to be generated exclusively by type I PI 3-kinases. Now, a novel route of PIP_3 synthesis, controlled by an unrelated enzyme family, has been discovered, increasing our understanding of the versatility of PIP_3 in cellular signalling.

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Current Biology 2001, 11:R371–R373

0960-9822/01/$ – see front matter

Since its discovery in the late 1980s [1,2], the phospholipid phosphatidylinositol(3,4,5)P_3 (PIP_3) has become established as an important mediator of signal transduction in numerous eukaryotic systems [3,4]. Although virtually undetectable in unstimulated cells, PIP_3 levels increase markedly in response to a variety of stimuli. Specific binding proteins are then recruited to the membrane and/or activated by their interaction with the lipid, contributing to the response to stimulation. Many different PIP_3-regulated proteins have been identified, including protein kinases, guanine nucleotide exchange factors and phospholipases. The ability of PIP_3 to interact with so many distinct targets suggests that its role in signalling is complex, different effectors being employed in distinct contexts to achieve appropriate responses.

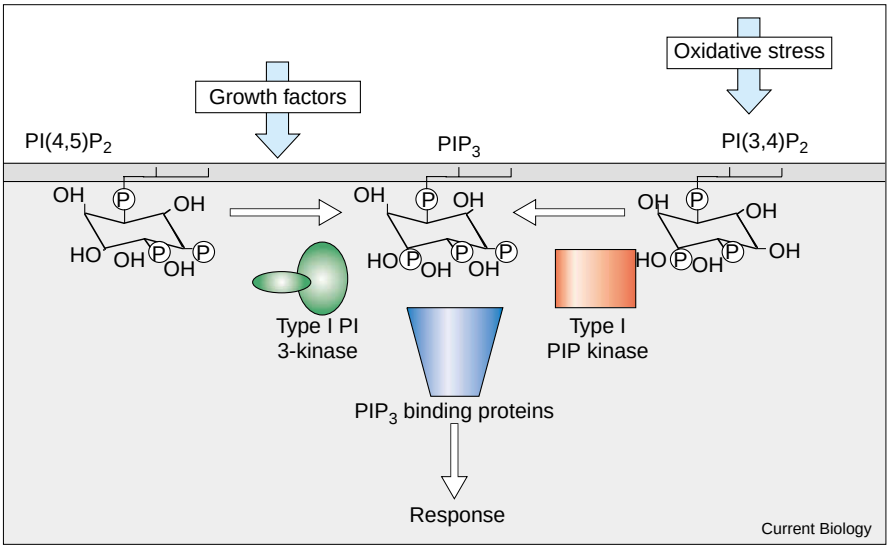
The PIP_3 generated in response to growth factors is produced by the phosphorylation of another phospholipid, PI(4,5)P_2, at position 3 of its inositol headgroup [5]. After the discovery of this pathway, numerous studies identified and characterised enzymes, the PI 3-kinases, responsible for its regulation (reviewed in [3,4]). Different PI 3-kinase isoforms are activated by distinct types of cell surface receptor, enabling PIP_3 to act as a mediator in a range of distinct signalling pathways. Cloning studies have shown these enzymes to be members of a wider family, all of which share the common ability to phosphorylate inositol at position 3, but which exhibit distinct preferences for their lipid substrate. These substrate preferences have led to the subdivision of the PI 3-kinase family into three classes, only class I enzymes being able to phosphorylate PI(4,5)P_2 and generate PIP_3 directly [3].

The intense interest focussed on the PI 3-kinases over the past decade has tended to overshadow the fact that these are not the only enzymes that phosphorylate inositol lipids. In fact, a distinct family of proteins lacking any discernible sequence homology with the PI 3-kinases also plays a major role in this process: the phosphatidylinositol phosphate kinases (PIP kinases; reviewed in [6]). These enzymes were initially identified as regulators of PI(4,5)P_2 synthesis, and this is clearly one of their major functions, but recent studies have shown them to utilise lipid substrates more promiscuously than originally realised [7,8]. This therefore raises the intriguing possibility that the PIP kinases might generate a wider range of products than initially suspected. In agreement with this idea, as reported recently in *Current Biology*, Halstead and co-workers [9] have now shown that one PIP kinase subclass, the type I isoforms, will generate PIP_3 *in vivo* without the involvement of type I PI 3-kinases, and that this reaction pathway is activated when cells are exposed to oxidative stress. In this case, type I PIP kinases synthesise PIP_3 by 5-phosphorylation of another member of the inositol lipid family, PI(3,4)P_2 (Figure 1).

PI(3,4)P_2 was initially identified in one of the studies that brought PIP_3 to prominence [2]. Subsequent analysis of the kinetics of its appearance within cells suggested that PI(3,4)P_2 is produced by dephosphorylation of PIP_3 [5], and a number of phosphatases able to catalyse this reaction are now known [10]. However, far from being merely an uninteresting breakdown product, it is now clear that PI(3,4)P_2 is functionally important in its own right. In particular, it can activate the protein kinase PKB/Akt, an important intermediary in numerous signalling pathways [11]. Unsurprisingly, and in keeping with its role as a signalling intermediate, PI(3,4)P_2 production can be regulated independently of the generation of PIP_3 ([12,13] and see below). It is presumably PI(3,4)P_2 synthesised by such a route that acts as the substrate of the type I PIP kinases for PIP_3 production.

The first indication that the type I PIP kinases have the potential to generate products other than PI(4,5)P_2 came from the finding that recombinant human type I PIP kinases can make PIP_3 *in vitro* [7]. This surprising result was subsequently confirmed by work on the murine type Iα and β PIP kinases, both of which synthesise PIP_3 *in vitro* when provided with PI(3,4)P_2 [8]. However, although *in vitro* phosphorylation reactions can be very informative, they do not always accurately reflect the processes regulated by particular enzymes physiologically: the type I PI 3-kinases enthusiastically phosphorylate several inositol lipids in the test tube, but seem only to utilise PI(4,5)P_2 *in vivo*. The new findings have addressed this problem, and show that, in agreement with their activities *in vitro*, type I PIP

Figure 1



Alternative pathways for the synthesis of PIP_3. The second messenger PIP_3 is generated either by the action of type I PI 3-kinase – activated, for example, by growth factor stimulation – or, by a newly discovered mechanism, via type I PIP kinase acting on $PI(3,4)P_2$. The latter, novel pathway is activated upon exposure of the cell to oxidative stress, possibly due to generation of $PI(3,4)P_2$ via an unidentified mechanism.

kinases are, in fact, able to phosphorylate $PI(3,4)P_2$ within living cells [9].

Halstead *et al.* [9] found that overexpression of type Iα PIP kinase, or of the related β and γ isoforms, in Cos-7 cells led to a marked increase in PIP_3 levels, achieved by 5-phosphorylation of $PI(3,4)P_2$. This increase is not seen if an inactive PIP kinase mutant is used, demonstrating an absolute requirement for the activity of this enzyme. Furthermore, PIP_3 synthesis in membranes from the transfected cells is insensitive to wortmannin, a potent inhibitor of type I PI 3-kinases, showing that it occurs independently of these proteins. Wortmannin-insensitive PIP_3 synthesis is also stimulated in membranes from cells exposed to oxidative stress by activation of an endogenous type I PIP kinase. These data therefore demonstrate that the type I PI 3-kinases are not the only enzymes capable of producing PIP_3 physiologically.

It is surprising that the type I PIP kinases contribute to signalling via PIP_3 production, as they also regulate levels of $PI(4,5)P_2$, itself a multifunctional lipid implicated in cellular processes as diverse as membrane trafficking and cytoskeletal regulation [6]. Significantly, levels of $PI(4,5)P_2$, as well as of PIP_3, are elevated in transfected cells producing exogenous type Iα PIP kinase [9], supporting the idea that this one enzyme uses multiple substrates *in vivo*. Arguably, the ability of a single enzyme to generate several functionally important products increases its potential utility to the cell, but this raises the question of how cells control which reaction is actually carried out.

Halstead and co-workers [9] found that an endogenous $PI(3,4)P_2$ 5-kinase is constitutively present in membranes from Cos-7 and MEL cells, but that in the absence of oxidative stress, PIP_3 is not produced unless exogenous $PI(3,4)P_2$ is supplied. The implication is that the PIP kinase responsible is not regulated by some unidentified signal promoting a change in its substrate preferences, but rather that PIP kinase-mediated PIP_3 synthesis may be regulated by controlling $PI(3,4)P_2$ availability. Interestingly, $PI(3,4)P_2$ production is stimulated by oxidative stress in Swiss 3T3 cells independently of PI 3-kinase activation [13], although in this cell line only a very modest increase in PIP_3 levels was detected. It is possible that this reflects cell-type specific differences in inositol lipid compartmentalisation, or type I PIP kinase expression, but this remains to be established.

As $PI(3,4)P_2$ is also functionally important in its own right, the question of how its levels are regulated is of great interest. In addition to its production by PIP_3 dephosphorylation, $PI(3,4)P_2$ can also be generated by phosphorylation of two further inositol lipids, PI(4)P and PI(3)P. The former reaction can be catalysed by type II PI 3-kinases, members of the PI 3-kinase superfamily that do not phosphorylate $PI(4,5)P_2$, and hence do not make PIP_3 [3]. In contrast, although evidence for an enzyme that catalyses 4-phosphorylation of PI(3)P has been around for some time [14, 15], its identity remains elusive. One potential candidate is type II PIP kinase, a near relative of the type I PIP kinases with distinct substrate preferences [6], which readily phosphorylates PI3P to $PI(3,4)P_2$ *in vitro* [7]. However, immunoprecipitation experiments using type II PIP kinase-specific antibodies showed a PI(3)P 4-kinase present in platelets to be a distinct enzyme [15]. Given these alternative possibilities, the source of the $PI(3,4)P_2$ converted to

PIP_3 by the type I PIP kinases must remain unclear for now, and future studies on the regulation of this novel pathway of PIP_3 synthesis should address this question.

What is the purpose of this unexpected complexity in cells' ability to regulate PIP_3 production? Clearly, cells have at their disposal a wider repertoire of pathways for the synthesis of this lipid than previously imagined, increasing yet further the range of circumstances under which it can be employed as a second messenger. Unfortunately, it is not yet known which of the many potential effectors of PIP_3 are employed downstream of its generation by PIP kinases, and how these differ from those utilised when PIP_3 is made by other means. Irrespective of our current lack of understanding of these factors, however, the fact remains that the versatility of PIP_3 as a signalling molecule highlights its central role in cellular regulation.

Acknowledgements

I am grateful to Robin Irvine for helpful discussions, and to the Medical Research Council for financial support.

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